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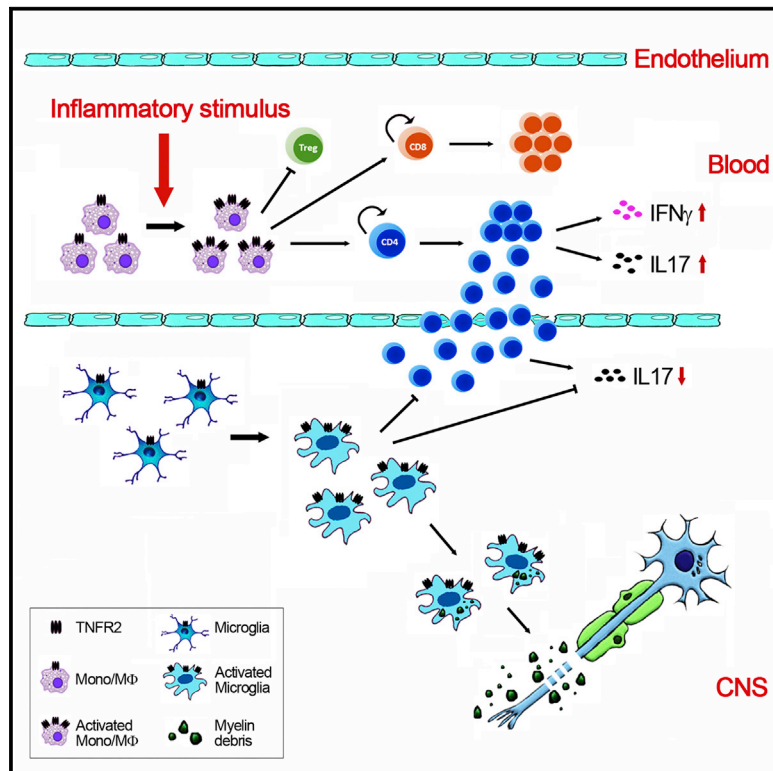
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Cell Reports

Opposing Functions of Microglial and Macrophagic TNFR2 in the Pathogenesis of Experimental Autoimmune Encephalomyelitis

Graphical Abstract



Authors

Han Gao, Matt C. Danzi, Claire S. Choi, ..., Vance P. Lemmon, Kate L. Lambertsen, Roberta Brambilla

Correspondence

r.brambilla@miami.edu

In Brief

Gao et al. uncover a dichotomy of functions for microglial versus monocyte/macrophagic TNFR2 in EAE pathophysiology. They demonstrate that TNFR2 in microglia is protective and provides signals to contain neuroinflammation, whereas TNFR2 in monocytes/macrophages is detrimental and drives immune activation and EAE initiation.

Highlights

- TNFR2 has opposing functions in microglia and monocytes/macrophages in EAE
- Microglial TNFR2 mediates protective responses at EAE onset
- Monocyte/macrophagic TNFR2 is detrimental in EAE by driving autoimmune activation

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Opposing Functions of Microglial and Macrophagic TNFR2 in the Pathogenesis of Experimental Autoimmune Encephalomyelitis

Han Gao,^{1,2} Matt C. Danzi,^{1,2,3} Claire S. Choi,⁴ Mehran Taherian,¹ Camilla Dalby-Hansen,^{1,5} Ditte G. Ellman,⁵ Pernille M. Madsen,^{1,5} John L. Bixby,^{1,2,3,6} Vance P. Lemmon,^{1,2,3} Kate L. Lambertsen,^{5,7,8} and Roberta Brambilla^{1,2,9,*}

¹The Miami Project to Cure Paralysis, Department of Neurological Surgery

²Neuroscience Program

³Center for Computational Science

University of Miami Miller School of Medicine, Miami, FL 33136, USA

⁴Columbia University, New York, NY 10027, USA

⁵Department of Neurobiology Research, Institute of Molecular Medicine, University of Southern Denmark, Odense C 5000, Denmark

⁶Department of Cellular and Molecular Pharmacology, University of Miami Miller School of Medicine, Miami, FL 33136, USA

⁷Brain Research - Inter-Disciplinary Guided Excellence, Department of Clinical Research, University of Southern Denmark, Odense C 5000, Denmark

⁸Department of Neurology, Odense University Hospital, Odense C 5000, Denmark

⁹Lead Contact

*Correspondence: r.brambilla@miami.edu

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SUMMARY

In multiple sclerosis (MS), soluble tumor necrosis factor (TNF) is detrimental via activation of TNF receptor 1 (TNFR1), whereas transmembrane TNF is beneficial primarily by activating TNF receptor 2 (TNFR2). Here, we investigate the role of TNFR2 in microglia and monocytes/macrophages in experimental autoimmune encephalomyelitis (EAE), a model of MS, by cell-specific gene targeting. We show that TNFR2 ablation in microglia leads to early onset of EAE with increased leukocyte infiltration, T cell activation, and demyelination in the central nervous system (CNS). Conversely, TNFR2 ablation in monocytes/macrophages results in EAE suppression with impaired peripheral T cell activation and reduced CNS T cell infiltration and demyelination. Our work uncovers a dichotomy of function for TNFR2 in myeloid cells, with microglial TNFR2 providing protective signals to contain disease and monocyte/macrophagic TNFR2 driving immune activation and EAE initiation. This must be taken into account when targeting TNFR2 for therapeutic purposes in neuroinflammatory diseases.

INTRODUCTION

Tumor necrosis factor (TNF) has been associated with the pathophysiology of multiple sclerosis (MS), because MS patients have high TNF concentrations in active lesions and cerebrospinal fluid, which correlate with the degree of disability (Hofman et al., 1989; Sharief and Hentges, 1991). TNF exists in two forms,

transmembrane (tmTNF) and soluble (solTNF). solTNF derives from cleavage of tmTNF by the TNF- α converting enzyme (TACE). tmTNF signals via TNF receptor 1 (TNFR1; *Tnfrsf1a*) and TNF receptor 2 (TNFR2; *Tnfrsf1b*), although with higher affinity for TNFR2, whereas solTNF signals via TNFR1 (Grell et al., 1995; Wajant et al., 2003). TNFR1 is widely expressed and mediates the majority of solTNF-dependent effects in inflammation, apoptosis, and neurotoxicity (Probert, 2015). Expression of TNFR2 is mostly restricted to immune and endothelial cells, where it sustains T regulatory (T_{reg}) function (Chen and Oppenheim, 2010), suppresses T helper (Th) 17 differentiation (Miller et al., 2015), and promotes lymphocyte proliferation (Tartaglia et al., 1993) and monocyte recruitment by endothelial cells (Venkatesh et al., 2013). In the central nervous system (CNS), TNFR2 is minimally expressed physiologically but is upregulated in microglia, astrocytes, and oligodendrocytes in neurological disease (Brambilla et al., 2011; Lambertsen et al., 2007). TNFR2 is virtually absent in CNS neurons, both in normal and pathological conditions (Brambilla et al., 2011; Probert, 2015), with motor neurons in a rodent model of Amyotrophic Lateral Sclerosis (ALS) (Veglianese et al., 2006) and a few neuron subsets in the Alzheimer's brain (Cheng et al., 2010) being the only exceptions reported. TNFR2 has been suggested to play a role in neuroprotection and remyelination (Arnett et al., 2001; Fontaine et al., 2002; Marchetti et al., 2004; Patel et al., 2012), but little is known about its function in individual CNS cell lineages in vivo. Recent studies from our lab have addressed this void by cell-specific gene-targeting approaches, demonstrating that oligodendroglial TNFR2 promotes oligodendrocyte differentiation and remyelination in experimental autoimmune encephalomyelitis (EAE), a model of MS (Madsen et al., 2016b). Microglia and monocytes/macrophages (Mo/M Φ s) play critical roles in MS and EAE (Shemer and Jung, 2015), and both express TNFR2 (Brambilla et al., 2011). However, the current knowledge of TNFR2 function

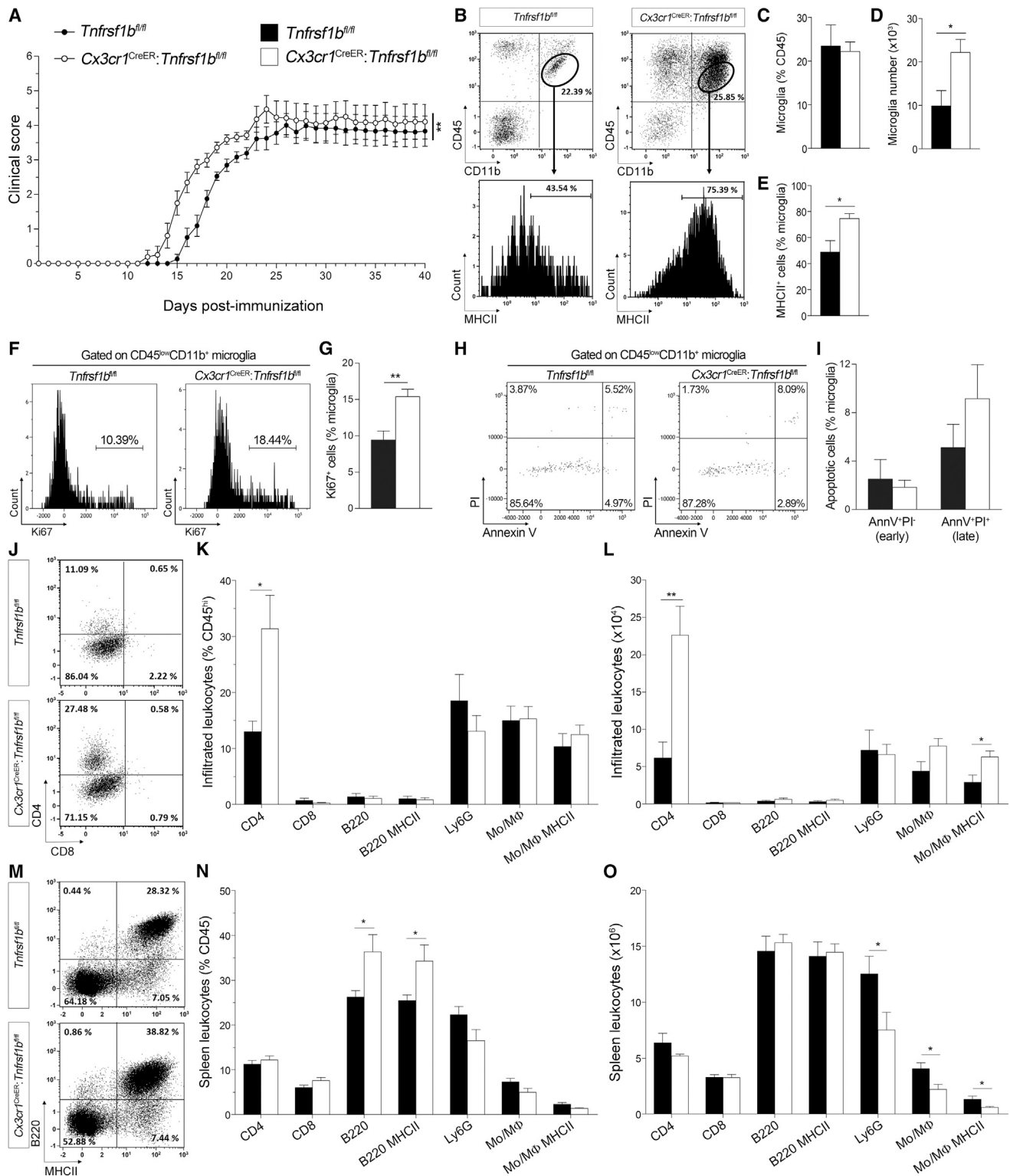


Figure 1. Ablation of Microglial TNFR2 in *Cx3cr1^{CreER};Tnfrsf1b^{fl/fl}* Mice Leads to the Early Onset of EAE

(A) Clinical course of EAE in *Tnfrsf1b^{fl/fl}* and *Cx3cr1^{CreER};Tnfrsf1b^{fl/fl}* mice. n = 13–17/group from two experiments; **p ≤ 0.01, one-way ANOVA, Mann-Whitney test.

(B–E) Flow cytometric analysis of microglia in the spinal cord at 17 dpi EAE. Quantification of percentage (C) and number (D) of microglia, and percentage of MHCII⁺ activated microglia (E). n = 5/group, *p ≤ 0.05, Student's t test.

(legend continued on next page)

in these populations is limited to only two in vitro studies: in microglia, TNFR2 was shown to promote the expression of anti-inflammatory and neuroprotective genes (Veroni et al., 2010), and in macrophages, to play an auxiliary role in activating proinflammatory TNFR1 signaling (Ruspi et al., 2014). Because TNFR2 function in microglia and Mo/MΦs in neurological disease is completely unknown, the goal of our study was to systematically dissect the roles of TNFR2 in these populations in EAE etiology. Using conditional knockout (cKO) models, we demonstrated that microglial TNFR2 is protective in the early stages of EAE, whereas monocyte/macrophagic TNFR2 is detrimental and drives disease initiation. Ablation of microglial TNFR2 accelerated EAE onset by establishing a proinflammatory environment that increased T cell activation in the CNS. In contrast, ablation of monocyte/macrophagic TNFR2 impaired T cell activation in the periphery, resulting in reduced CNS immune cell infiltration and EAE suppression. Taken together, our data demonstrate the opposite functions of TNFR2 signaling in microglia and peripheral Mo/MΦs in the pathophysiology of EAE. This dichotomy needs to be addressed when targeting TNFR2 signaling for therapeutic purposes in MS or any other neuroinflammatory disease in which both cell populations are major contributors.

RESULTS

Ablation of Microglial TNFR2 in *Cx3cr1^{CreER}:Tnfrsf1b^{fl/fl}* Mice Leads to the Early Onset of EAE

Selective gene targeting in microglia has been challenging because of their shared gene profile with Mo/MΦs. To restrict *Tnfrsf1b^{fl/fl}* ablation to microglia, we generated *Cx3cr1^{CreER}:Tnfrsf1b^{fl/fl}* cKO mice and adopted an established tamoxifen (tam) induction protocol that exploits the different origins and turnover rates of microglia versus other CX₃CR₁⁺ myeloid cells (Goldmann et al., 2013; Parkhurst et al., 2013). In this protocol, five daily tam injections are followed by a 28-day waiting period (instead of the usual 5–7 days) during which fast-renewing Mo/MΦs are replaced by wild-type (WT) cells derived from bone marrow precursors, whereas self-renewing microglia are not replaced and maintain *Tnfrsf1b* ablation. Even though specific microglial gene targeting has already been demonstrated with this protocol (Goldmann et al., 2013; Parkhurst et al., 2013), we still validated it in our own setting by comparison with a conventional 5-day waiting period. To do so, we crossed *Cx3cr1^{CreER}* mice, which constitutively express enhanced yellow fluorescent protein (EYFP) in CX₃CR₁⁺ cells, with *Rosa26^{tdTomato/+}* reporters. In the spinal cord we detected recombination by tdTomato fluorescence in almost 90% of EYFP⁺ microglia (Fig-

ures S1A and S1C). In the spleen, recombination was decreased from 40% to about 3% in EYFP⁺ myeloid cells 28 days after tam injection, confirming that gene targeting was restricted to CX₃CR₁⁺ microglia and not Mo/MΦs (Figures S1B and S1C). TNFR2 was barely present in microglia in naive conditions (less than 5%; Figures S2A–S2C) but was robustly upregulated after EAE (Figures S2D–S2J) to about 70% in *Tnfrsf1b^{fl/fl}* mice and was reduced to 30% in *Cx3cr1^{CreER}:Tnfrsf1b^{fl/fl}* mice (Figures S2D and S2E). Both splenic and CNS-infiltrated Mo/MΦs highly expressed TNFR2 after EAE (Figures S2F–S2I) with no difference between genotypes. These data demonstrate that *Cx3cr1^{CreER}:Tnfrsf1b^{fl/fl}* mice induced with the 28-day tam protocol are an excellent model for selective TNFR2 ablation in microglia. Analysis with Rotorod and open-field tests showed no abnormal locomotor phenotype (Figures S3A–S3C) or altered spontaneous activity (Figures S3D–S3H) in naive *Cx3cr1^{CreER}:Tnfrsf1b^{fl/fl}* mice. Furthermore, microglial number was comparable with controls (Figure S3I), and so was the expression of microglia-associated genes, such as *Trem2* and *P2ry12* (Figure S3J). Notably, microglial *Tnfrsf1b*, which is minimally expressed in naive microglia (Figure S2A), did not change between *Tnfrsf1b^{fl/fl}* and *Cx3cr1^{CreER}:Tnfrsf1b^{fl/fl}* mice, which explains the lack of phenotypical alterations in naive animals.

Induced with EAE, *Cx3cr1^{CreER}:Tnfrsf1b^{fl/fl}* mice displayed earlier disease onset, although the clinical course later overlapped that of *Tnfrsf1b^{fl/fl}* controls (Figure 1A; Table S1). At 17 days post induction (dpi), when the highest difference in clinical scores was observed, *Cx3cr1^{CreER}:Tnfrsf1b^{fl/fl}* mice showed more robust microglial activation. Indeed, CD45^{low}CD11b⁺ total microglia (Figures 1B–1D) and major histocompatibility complex II⁺ (MHCII⁺) activated microglia (Figures 1B and 1E) were significantly increased. This was due to enhanced proliferation, as shown by the higher number of Ki67⁺ microglia (Figures 1F and 1G), and not to an effect on survival, because the numbers of propidium iodide (PI[−]) Annexin V⁺ early apoptotic and PI⁺ Annexin V⁺ late apoptotic and necrotic microglia were comparable between genotypes (Figures 1H and 1I). Analysis of immune cell infiltration into the spinal cord showed a marked increase in CD4 T cells and MHCII⁺ activated Mo/MΦs in *Cx3cr1^{CreER}:Tnfrsf1b^{fl/fl}* mice (Figures 1J–1L), which correlated with the earlier disease symptoms. Ly6G⁺ neutrophils and total and MHCII⁺ Mo/MΦs (Figure 1K) were significantly lower, likely because of faster mobilization into circulation. Notably, *Cx3cr1^{CreER}:Tnfrsf1b^{fl/fl}* mice had higher frequency of total and MHCII⁺ splenic B cells (Figures 1M and 1N), which indicates increased antigen presentation capacity and could explain, at least in part, the increased expansion of CD4 T cells.

(F) Representative flow plots of Ki67 expression in microglia at 17 dpi.

(G) Quantification of Ki67⁺ microglia. n = 4–5/group; **p ≤ 0.01, Student's t test.

(H) Representative flow plots of Annexin V expression in microglia at 17 dpi.

(I) Quantification of Annexin V⁺PI[−] and Annexin V⁺PI⁺ microglia. n = 4–5/group.

(J) Representative flow plots of CD4 and CD8 cells in the spinal cord at 17 dpi.

(K and L) Percentages (K) and absolute numbers (L) of infiltrated immune cells. Mo/MΦs are defined as CD45^{hi}CD11b⁺NK1.1[−]Ly6G[−]SSA^{low}. n = 5/group; *p ≤ 0.05, **p ≤ 0.01, Student's t test.

(M) Representative flow plots of total and MHCII⁺ splenic B cells at 17 dpi.

(N and O) Percentages (N) and absolute numbers (O) of splenic immune cells. n = 6/group; *p ≤ 0.05, Student's t test.

All graph bars represent SEM.

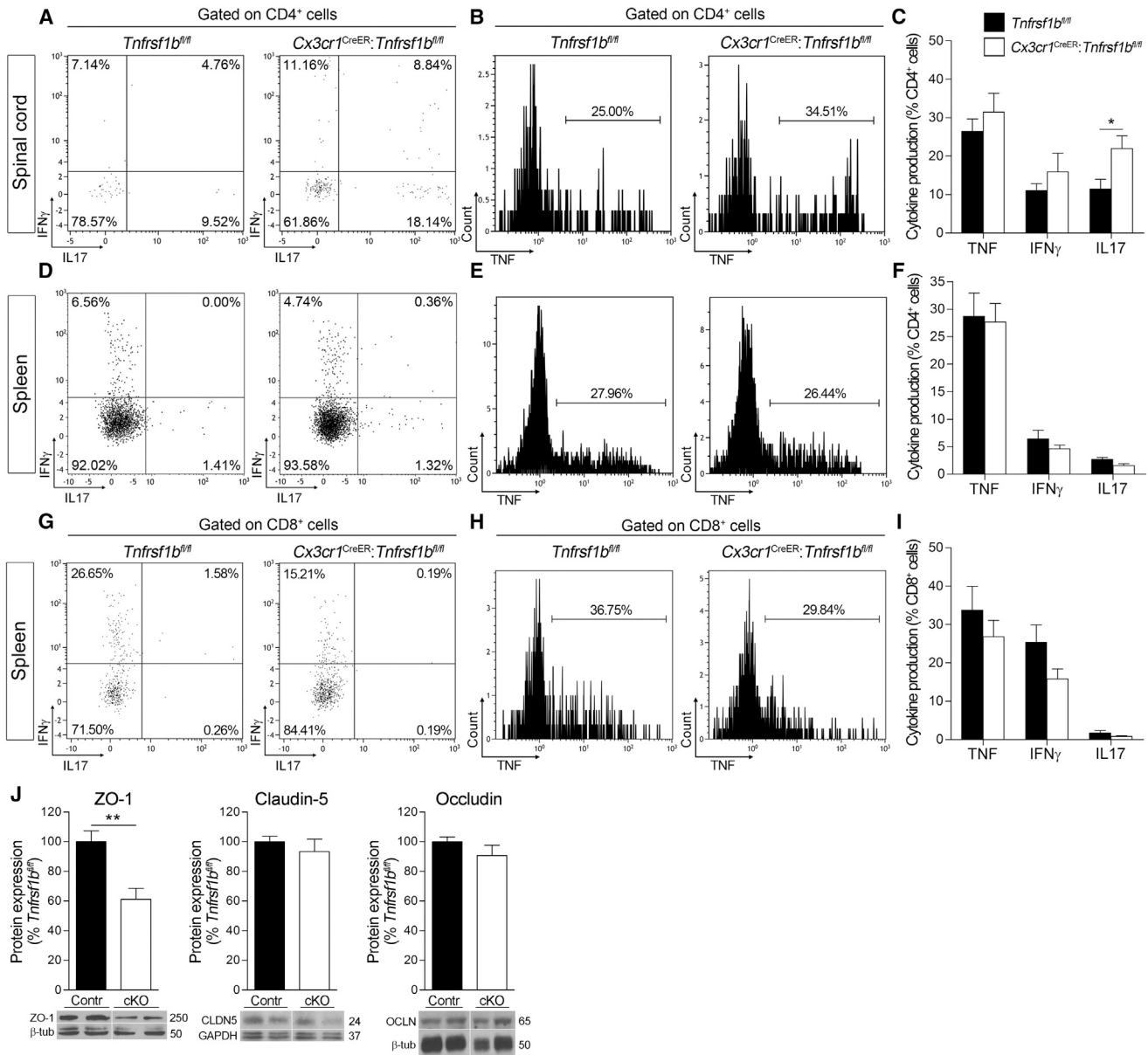


Figure 2. Ablation of Microglial TNFR2 Leads to Exacerbated T Cell Effector Function in the Spinal Cord after EAE

(A, B, D, E, G, and H) Representative flow plots of cytokine expression in CD4 (A, B, D, E) and CD8 (G, H) T cells isolated from spinal cord and spleen of *Tnfrsf1b*^{fl/fl} and *Cx3cr1*^{CreER}:*Tnfrsf1b*^{fl/fl} mice at 17 dpi; n = 4–5/group; *p ≤ 0.05, Student's t test.

(C, F, and I) Quantification of cytokine expression in CD4 (C, F) and CD8 (I) T cells from spinal cord and spleen; n = 4–5/group.

(J) Western blot analysis of tight junction proteins in the spinal cord at 12 dpi EAE; n = 4–9/group; **p ≤ 0.01, Student's t test.

All graph bars represent SEM.

Ablation of Microglial TNFR2 Leads to Exacerbated T Cell Effector Function in the Spinal Cord after EAE

Because EAE symptoms are largely driven by activated T cells, we tested T effector function by measuring Th1 and Th17 cytokines after ex vivo restimulation. In the spinal cord, infiltrated CD4 cells from *Cx3cr1*^{CreER}:*Tnfrsf1b*^{fl/fl} mice showed increased interleukin (IL)-17 production (Figures 2A and 2C), but no changes in interferon γ (IFN γ) and TNF (Figures 2A–2C). No differences were found in splenic CD4 and CD8 cells (Figures 2D–2I). This suggests

that TNFR2-ablated microglia establish an environment prone to driving T cells toward Th17 differentiation in the CNS. Interestingly, prior to disease onset (12 dpi), *Cx3cr1*^{CreER}:*Tnfrsf1b*^{fl/fl} mice showed reduced expression of the tight junction protein Zona Occludens-1 (ZO-1) in the spinal cord (Figure 2J). By binding to other tight junction elements like Claudin-5 and Occludin, which did not change in our model (Figure 2J), ZO-1 plays a crucial role in blood-brain barrier (BBB) integrity (Bennett et al., 2010), and its downregulation is indicative of increased BBB permeability, which

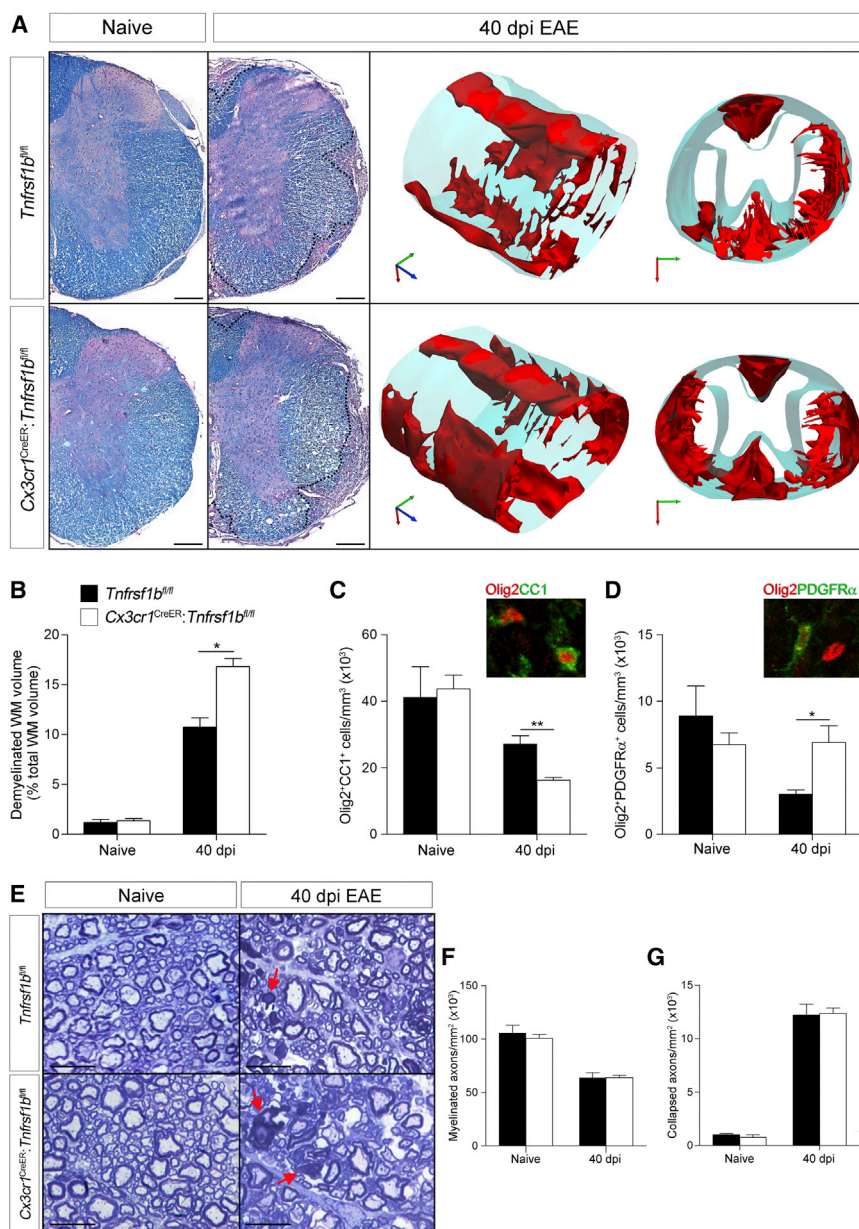


Figure 3. Microglial TNFR2 Ablation Leads to Increased Demyelination with Chronic EAE

(A) Assessment of demyelination in the spinal cord of *Tnfrsf1b^{fl/fl}* and *Cx3cr1^{CreER};Tnfrsf1b^{fl/fl}* mice by LFB staining. Left: contoured areas show regions of demyelinated white matter. Scale bars, 200 μ m. Right: 3D reconstructions of the demyelinated areas (red).

(B) Quantification of the demyelinated white matter volume. $n = 3-5$ /group, $*p \leq 0.05$, Student's *t* test.

(C and D) Quantification of Olig2⁺CC1⁺ cells (C) and Olig2⁺PDGFRα⁺ cells (D) in the spinal cord. $n = 3-5$ /group; $*p \leq 0.05$, $**p \leq 0.01$, Student's *t* test.

(E) Toluidine blue staining of spinal cord sections from naive and 40 dpi mice. Red arrows indicate degenerated collapsed axons. Scale bars, 10 μ m. (F and G) Quantification of myelinated (F) and degenerated (G) axons. $n = 4-5$ /group. All graph bars represent SEM.

into mature oligodendrocytes, because TNFR2 has been shown to regulate this process (Madsen et al., 2016b). Analysis of axonal pathology showed no difference in the number of intact (Figures 3E and 3F) or degenerated axons (Figures 3E and 3G). This may explain the similar clinical scores at chronic disease despite myelin damage being more severe in *Cx3cr1^{CreER};Tnfrsf1b^{fl/fl}* mice.

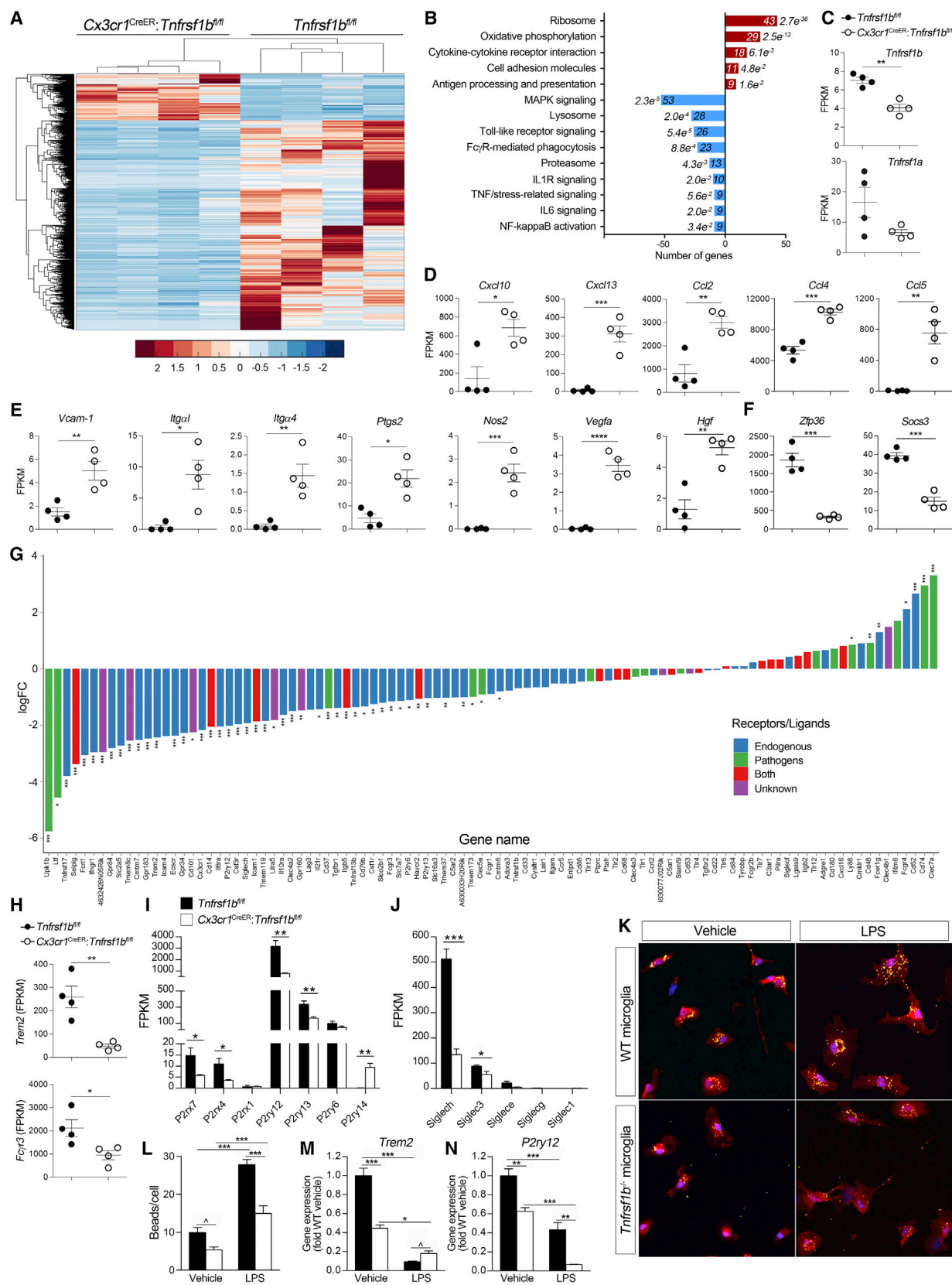
TNFR2-Ablated Microglia Develop a Proinflammatory Phenotype with Dysregulated Expression of Homeostatic and Host Defense Genes after EAE

To dissect the mechanisms by which microglial TNFR2 regulates EAE pathogenesis, we analyzed the transcriptome of spinal cord microglia from *Tnfrsf1b^{fl/fl}* and *Cx3cr1^{CreER};Tnfrsf1b^{fl/fl}* mice at 17 dpi using RNA sequencing (RNA-seq) (GEO: GSE78082). We found 5,049 differentially expressed genes with high reproducibility across samples (Figure 4A); 20% were upregulated (1,022 genes) and 80% downregulated (4,027 genes) in *Cx3cr1^{CreER};Tnfrsf1b^{fl/fl}* compared with *Tnfrsf1b^{fl/fl}* mice (Figure 4A), including *Tnfrsf1b* (Figure 4C). Interestingly, *Tnfrsf1a* did not change (Figure 4C; Figure S4A), indicating that microglial TNFR2 does not have a modulatory function on TNFR1 at the transcriptional level. Changes were validated by qPCR on select genes and matched the RNA-seq data (Figure S4A). Most of the downregulated genes belonged to pathways controlling cell homeostasis, such as mitogen-activated protein kinase (MAPK) and nuclear factor κ B (NF- κ B) (Figure 4B), and were found to be physically or functionally connected with TNFR2 (Figure S4B). Notably, the dysregulated genes included modulators of two key aspects of microglia innate immune function: inflammation and

could contribute to the increased immune cell infiltration in *Cx3cr1^{CreER};Tnfrsf1b^{fl/fl}* mice (Figure 1L).

Ablation of Microglial TNFR2 Leads to Increased Demyelination with Chronic EAE

Even though *Cx3cr1^{CreER};Tnfrsf1b^{fl/fl}* and *Tnfrsf1b^{fl/fl}* mice showed overlapping clinical profiles at chronic EAE, analysis of the spinal cord at 40 dpi revealed increased white matter damage in *Cx3cr1^{CreER};Tnfrsf1b^{fl/fl}* mice (Figures 3A and 3B). This was paralleled by higher loss of Olig2⁺CC1⁺ oligodendrocytes (Figure 3C) and an increase in Olig2⁺PDGFRα⁺ oligodendrocyte precursor cells (OPCs) (Figure 3D). Increased OPC numbers could depend on increased proliferation and survival to repair myelin damage (Maier et al., 2013), or on impaired differentiation



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host defense. With respect to inflammation, TNFR2-ablated microglia showed upregulation of chemokines (Figure 4D), cell adhesion molecules, oxidative enzymes, and growth factors (Figure 4E), and downregulation of antiinflammatory signals like *Zfp36* and *Socs3* (Figure 4F). With respect to host defense, microglia from *Cx3cr1^{CreER}:Tnfrsf1b^{fl/fl}* mice showed dysregulation of the “microglial sensome” (Hickman et al., 2013), a set of molecules that define the microglial surveillance machinery. Only 7.1% of sensome genes were upregulated in *Cx3cr1^{CreER}:Tnfrsf1b^{fl/fl}* mice and more than 46% were downregulated (Figure 4G). These include receptors for pathogen recognition (*Fcγr3*), phagocytosis (*Trem2*) (Figure 4H), and tissue surveillance (purinergic and Siglec receptors; Figures 4I and 4J), suggesting that without TNFR2, key homeostatic functions of microglia may be altered. Phagocytosis was indeed compromised, because cultured microglia from *Tnfrsf1b^{-/-}* mice showed a reduced capacity to engulf fluorescent beads both in unstimulated and in lipopolysaccharide (LPS)-stimulated conditions (Figures 4K and 4L). Importantly, *Trem2* and *P2ry12* were reduced in *Tnfrsf1b^{-/-}* unstimulated microglia (Figures 4M and 4N) and further downregulated in both genotypes after LPS stimulation. This suggests that TNFR2 activation is necessary for constitutive expression of these genes. *Trem2* and *P2ry12* were also assessed after stimulation with *soITNF*, which activates proinflammatory TNFR1. Unlike LPS, *soITNF* did not maintain suppression of *Trem2* and *P2ry12* in *Tnfrsf1b^{-/-}* microglia (Figure S4C). This indicates that transcriptional regulation of *Trem2* and *P2ry12* by *soITNF* depends not only on direct TNFR1 activation, but also on the auxiliary presence of TNFR2. The cooperative signaling of TNFR1 and TNFR2 in myeloid cells also has been suggested in previous reports (Ruspi et al., 2014).

Collectively, these data indicate that without TNFR2, microglia develop a more invasive proinflammatory phenotype that accelerates EAE pathogenesis, while losing signals necessary to carry out essential homeostatic functions, including tissue surveillance and host defense.

Ablation of TNFR2 in *LysM^{Cre}:Tnfrsf1b^{fl/fl}* Mice Results in Suppression of EAE

To assess TNFR2 function in Mo/MΦs, we used *LysM^{Cre}:Tnfrsf1b^{fl/fl}* cKOs in combination with a bone marrow transplantation strategy. To start, we tested *LysM^{Cre}* recombination efficiency in peripheral and CNS myeloid cells by crossing

LysM^{Cre+/-} mice with *Rosa26^{tdTomato+/-}* reporters. In splenic myeloid cells, recombination was similar in naive and EAE conditions reaching approximately 50% (Figures S5A and S5C). In spinal cord microglia, recombination was inefficient in naive conditions, with only 10% of CD11b⁺CX₃CR₁⁺ cells positive for tdTomato, which increased to 30% after EAE (Figures S5B and S5D). TNFR2 expression, virtually absent in naive conditions (Figures S2A and S2B), amounted to more than 60% of splenic Mo/MΦs and microglia in *Tnfrsf1b^{fl/fl}* mice after EAE, and was significantly reduced in both populations in *LysM^{Cre}:Tnfrsf1b^{fl/fl}* mice (Figures S5E, S5F, S5I, and S5J; Table S2). This was confirmed by immunohistochemistry (Figure S5K). We also confirmed that TNFR2 ablation did not interfere with TNFR1 expression in splenic Mo/MΦs (Figures S5G and S5H). Because the *LysM* promoter is active in granulocytes and other immune cells (Goldmann et al., 2013), to assess specificity of myeloid ablation, we measured TNFR2 expression in various splenic populations (Table S2). TNFR2 was present in T cells, B cells, and neutrophils with no difference between *Tnfrsf1b^{fl/fl}* and *LysM^{Cre}:Tnfrsf1b^{fl/fl}* mice. In NKs, TNFR2 was upregulated after EAE, with mild but significant reduction in *LysM^{Cre}:Tnfrsf1b^{fl/fl}* mice. This should not influence the *LysM^{Cre}:Tnfrsf1b^{fl/fl}* phenotype because NKs represent only a small fraction of the splenic population (less than 3%; Figure 5E). It is worth noting that neuronal expression of Cre recombinase in *LysM^{Cre}* mice has been reported by various groups including ours (Clausen et al., 2016; Orthgiess et al., 2016). However, because TNFR2 is virtually absent in CNS neurons (Brambilla et al., 2011; Zhang et al., 2014), neuronal Cre activity should not affect TNFR2 expression in *LysM^{Cre}:Tnfrsf1b^{fl/fl}* mice. Like *Cx3cr1^{CreER}:Tnfrsf1b^{fl/fl}* mice, *LysM^{Cre}:Tnfrsf1b^{fl/fl}* mice did not show alterations in locomotor function, spontaneous activity, or splenic leukocyte profile (Figure S6).

To test myeloid TNFR2 function, we induced EAE in *Tnfrsf1b^{fl/fl}* and *LysM^{Cre}:Tnfrsf1b^{fl/fl}* mice. Contrary to *Cx3cr1^{CreER}:Tnfrsf1b^{fl/fl}* cKOs, EAE was markedly suppressed in *LysM^{Cre}:Tnfrsf1b^{fl/fl}* mice, with average scores less than 2 (flaccid tail, but no paralysis) (Figure 5A; Table S3). Because TNFR2 ablation occurs in both Mo/MΦs and microglia in this model, to dissect the contribution of each population to EAE development, we generated bone marrow chimeras with gene ablation specific to either Mo/MΦs (*LysM^{Cre}:Tnfrsf1b^{fl/fl}* → CD45.1) or microglia (CD45.1 → *LysM^{Cre}:Tnfrsf1b^{fl/fl}*). In *LysM^{Cre}:Tnfrsf1b^{fl/fl}* → CD45.1

Figure 4. RNA-Seq Analysis of the Microglial Transcriptome after EAE

(A) Heatmap of differentially expressed genes in spinal cord microglia sorted from *Cx3cr1^{CreER}:Tnfrsf1b^{fl/fl}* and *Tnfrsf1b^{fl/fl}* mice at 17 dpi; n = 4/group. Changes are expressed as log₂ values. Red represents upregulated genes; blue represents downregulated genes.
(B) DAVID pathway analysis of differentially expressed genes in *Cx3cr1^{CreER}:Tnfrsf1b^{fl/fl}* versus *Tnfrsf1b^{fl/fl}* mice. Red represents upregulated pathways; blue represents downregulated pathways. The p values are shown next to the bars.
(C–F and H) Gene expression profiles of TNF receptors (C), chemokines (D), proinflammatory molecules/growth factors (E), anti-inflammatory mediators (F), and sensome genes (H) from the RNA-seq analysis shown as fragments per kilobase per million mapped reads (FPKM); *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001, Student's t test.
(G) Expression of microglial sensome genes in *Cx3cr1^{CreER}:Tnfrsf1b^{fl/fl}* versus *Tnfrsf1b^{fl/fl}* mice. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, EdgeR analysis.
(I and J) Expression of purinergic (I) and Siglec (J) receptors obtained from the RNA-seq analysis. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, Student's t test.
(K and L) Quantification of microglial phagocytosis in vitro. Fluorescent beads incorporated by primary microglia from WT and *Tnfrsf1b^{-/-}* mice (K) were quantified in unstimulated conditions or after LPS treatment (L). n = 6/group; ***p ≤ 0.001, one-way ANOVA, Tukey test; *p ≤ 0.05, Student's t test.
(M and N) Expression of *Trem2* (M) and *P2ry12* (N) in cultured microglia. n = 6/group; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, one-way ANOVA, Tukey test; *p ≤ 0.05, Student's t test.
All graph bars represent SEM. See also Figure S4.

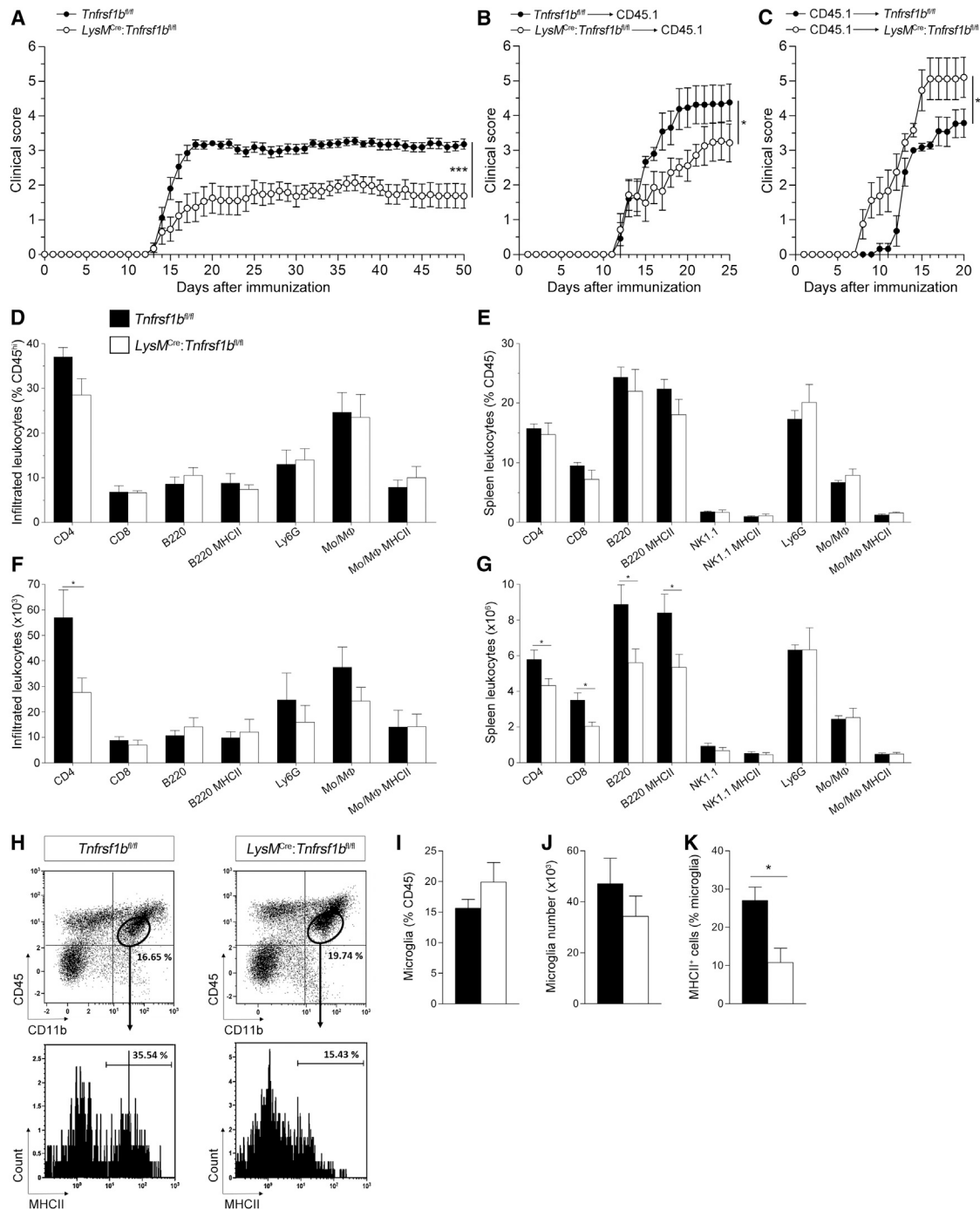


Figure 5. Ablation of TNFR2 in *LysM*^{Cre};*Tnfrsf1b*^{fl/fl} Mice Results in Suppression of EAE

(A) Clinical course of EAE in *Tnfrsf1b*^{fl/fl} and *LysM*^{Cre};*Tnfrsf1b*^{fl/fl} mice. n = 12–13/group from two experiments; ***p < 0.001, one-way ANOVA, Mann-Whitney test. (B and C) Clinical course of EAE in bone-marrow-transplanted chimeric mice with TNFR2 ablation in Mo/MΦs (*LysM*^{Cre};*Tnfrsf1b*^{fl/fl} → CD45.1) and corresponding controls (*Tnfrsf1b*^{fl/fl} → CD45.1) (B), or in microglia (CD45.1 → *LysM*^{Cre};*Tnfrsf1b*^{fl/fl}) and corresponding controls (CD45.1 → *Tnfrsf1b*^{fl/fl}) (C). n = 6–7/group; *p < 0.05, one-way ANOVA, Mann-Whitney test. (D and F) Flow cytometric analysis of the frequency (D) and absolute numbers (F) of infiltrated leukocytes at 20 dpi. n = 5/group; *p < 0.05, Student's t test. (E and G) Flow cytometric analysis of the frequency (E) and absolute numbers (G) of splenic leukocytes at 20 dpi. Mo/MΦs are defined as CD45^{hi}CD11b⁺NK1.1[−]Ly6G[−]SSA^{low}. n = 8–11/group; *p < 0.05, Student's t test. (H) Representative flow plots of spinal cord microglia at 20 dpi. (I–K) Quantification of percentage (I) and number (J) of microglia, and percentage of MHCII⁺ activated microglia (K). n = 5–6/group; *p < 0.05, Student's t test. All graph bars represent SEM.

mice, EAE development and progression were suppressed (Figure 5B), mirroring the profile of *LysM^{Cre}:Tnfrsf1b^{fl/fl}* mice (Figure 5A). Conversely, in *CD45.1→LysM^{Cre}:Tnfrsf1b^{fl/fl}* mice, EAE had accelerated onset and more severe progression (Figure 5C), similarly to *Cx3cr1^{CreER}:Tnfrsf1b^{fl/fl}* mice (Figure 1A). At acute disease (20 dpi), the percentages of splenic cell populations in *LysM^{Cre}:Tnfrsf1b^{fl/fl}* mice were comparable with *Tnfrsf1b^{fl/fl}* mice (Figure 5E), but the numbers of CD4 and CD8 T cells, B cells, and MHCII⁺ activated B cells were reduced (Figure 5G). The percentages of CNS-infiltrated cells were also unchanged (Figure 5D), but the absolute number of CD4 T cells was markedly lower (Figure 5F). In addition, the microglial response was assessed, and although we did not see changes in cell number, we detected a reduction in the frequency of MHCII⁺ activated microglia in *LysM^{Cre}:Tnfrsf1b^{fl/fl}* mice (Figures 5H–5K), which could be a consequence of the reduced presence of proinflammatory CD4 cells in the spinal cord. These results indicate that TNFR2 ablation in Mo/MΦs and not microglia accounts for EAE suppression in *LysM^{Cre}:Tnfrsf1b^{fl/fl}* mice, suggesting that TNFR2 in peripheral myeloid cells is required for EAE induction.

Ablation of TNFR2 in *LysM^{Cre}:Tnfrsf1b^{fl/fl}* Mice Results in Impaired T Cell Proliferation and Effector Function in the Spleen after EAE

T cell expansion and activation are key for EAE initiation (Sospedra and Martin, 2005). Because we found reduced splenic T and B cells, and reduced infiltrated CD4 T cells in *LysM^{Cre}:Tnfrsf1b^{fl/fl}* mice (Figures 5F and 5G), we investigated whether TNFR2 ablation from Mo/MΦs impaired lymphocyte proliferation and activation preventing EAE induction. In the spleen, we analyzed proliferation at pre-disease (12 dpi) by Ki67 labeling and found in *LysM^{Cre}:Tnfrsf1b^{fl/fl}* mice a reduction in Ki67⁺ CD4 and CD8 T cells, but no difference in B cells (Figures 6A–6C). Effector function was assessed at acute disease (20 dpi) by measuring Th1 and Th17 cytokines. In splenic CD4 T cells, TNF production did not change (Figure S7A), but IFN γ and IL-17 were reduced in *LysM^{Cre}:Tnfrsf1b^{fl/fl}* mice (Figures 6D and 6E). No differences were found in the CD8 population (Figures S7B and S7C). On the contrary, IL-17 was increased in infiltrated CD4 cells of *LysM^{Cre}:Tnfrsf1b^{fl/fl}* mice (Figures 6D and 6G), with no difference in CD8 cells (Figure 6H; Figure S7E). IFN γ and TNF did not change (Figures S7D–S7F). Interestingly, *Cx3cr1^{CreER}:Tnfrsf1b^{fl/fl}* mice showed a similar profile (Figure 2A), suggesting that even minimal ablation of microglial TNFR2 as in *LysM^{Cre}:Tnfrsf1b^{fl/fl}* mice may be sufficient to alter the CNS environment and promote Th17 differentiation.

We went further to test whether reduced T cell expansion and activation in *LysM^{Cre}:Tnfrsf1b^{fl/fl}* mice could be associated with increased presence of regulatory cells in the spleen. We did not detect any changes in IL-10–producing B1a and B1b B regulatory cells (B_{regs}) (Figures S7G and S7H), but we observed an increase in the frequency of CD25⁺FoxP3⁺ T_{regs} (Figures 6I and 6J), which could contribute to the suppressed T cell response.

Together these data indicate that ablation of TNFR2 in peripheral Mo/MΦs compromises T cell activation and function resulting in inability to mount an efficient immune response and initiate EAE.

Ablation of TNFR2 in *LysM^{Cre}:Tnfrsf1b^{fl/fl}* Mice Results in Myelin Preservation and Neuroprotection in EAE

To investigate whether the improved functional outcome in *LysM^{Cre}:Tnfrsf1b^{fl/fl}* mice corresponded to reduced myelin and axon pathology, we assessed white matter damage in the spinal cord by Luxol fast blue (LFB) staining. At 50 dpi, *LysM^{Cre}:Tnfrsf1b^{fl/fl}* mice showed reduced demyelination (Figures 7A and 7B), accompanied by a higher number of Olig2⁺PDGFR α ⁺ OPCs (Figure 7C). Electron microscopy (EM) analysis of remyelination showed *LysM^{Cre}:Tnfrsf1b^{fl/fl}* mice to have a higher number of remyelinated axons in the spinal cord (Figures 7D and 7E), which, together with the higher presence of OPCs, suggests that repair mechanisms are more efficient in these mice. Evaluation of axonal damage showed significant neuroprotection in *LysM^{Cre}:Tnfrsf1b^{fl/fl}* mice, with more intact axons (Figures 7F and 7G) and reduced accumulation of degenerated axons (Figures 7F and 7H), which correlated with the lower clinical scores at chronic EAE.

DISCUSSION

In the present study we uncover a dichotomy of functions for microglial versus monocyte/macrophagic TNFR2 in EAE pathophysiology. We demonstrate that TNFR2 in microglia is protective by providing signals to contain neuroinflammation, whereas TNFR2 in Mo/MΦs is detrimental by driving immune activation and EAE initiation.

Microglia and Mo/MΦs are the main effectors of the innate immune response and, as such, perform homeostatic and surveillance functions sharing similar roles in different compartments, the CNS or the periphery (Ginhoux and Jung, 2014; Prinz et al., 2014). This explains why much of the molecular machinery is common to the two populations, despite their distinct developmental origin (Ginhoux et al., 2010). Exceptions to this rule are signature genes that have been recently identified as primarily expressed in one or the other, such as *P2ry12* and *Cx3cr1* found almost exclusively in microglia, and *P2rx4*, *Ccr2*, and *Ifitm* genes found uniquely in Mo/MΦs (Hickman et al., 2013). Capitalizing on this genetic diversity and developmental origin, the *Cx3cr1^{CreER/+}* mouse line has allowed to specifically target microglia when using a tamoxifen induction protocol devised to take advantage of the different turnover rates of myeloid cells (Goldmann et al., 2013; Parkhurst et al., 2013; Yona et al., 2013). With this strategy we generated *Cx3cr1^{CreER}:Tnfrsf1b^{fl/fl}* mice to dissect the role of microglial TNFR2 in EAE. Comparative transcriptome analysis shows that microglia are the neural cells with the highest expression of TNFR2 (http://web.stanford.edu/group/barres_lab/cgi-bin/igv.cgi_2.py?lname=Tnfrsf1b) (Zhang et al., 2014). Because tmTNF, the activating ligand of TNFR2, is protective in EAE (Brambilla et al., 2011; Taoufik et al., 2011), and in vitro evidence showed that TNFR2 is able to activate antiinflammatory signals (Veroni et al., 2010), we hypothesized that microglial TNFR2 could be the executor, at least in part, of tmTNF beneficial functions in vivo. In support of this hypothesis, *Cx3cr1^{CreER}:Tnfrsf1b^{fl/fl}* mice developed EAE earlier, showing signs of paralysis more than 2 days in advance of *Tnfrsf1b^{fl/fl}* controls. This was accompanied by elevated numbers and cellular activation of microglia at disease onset,

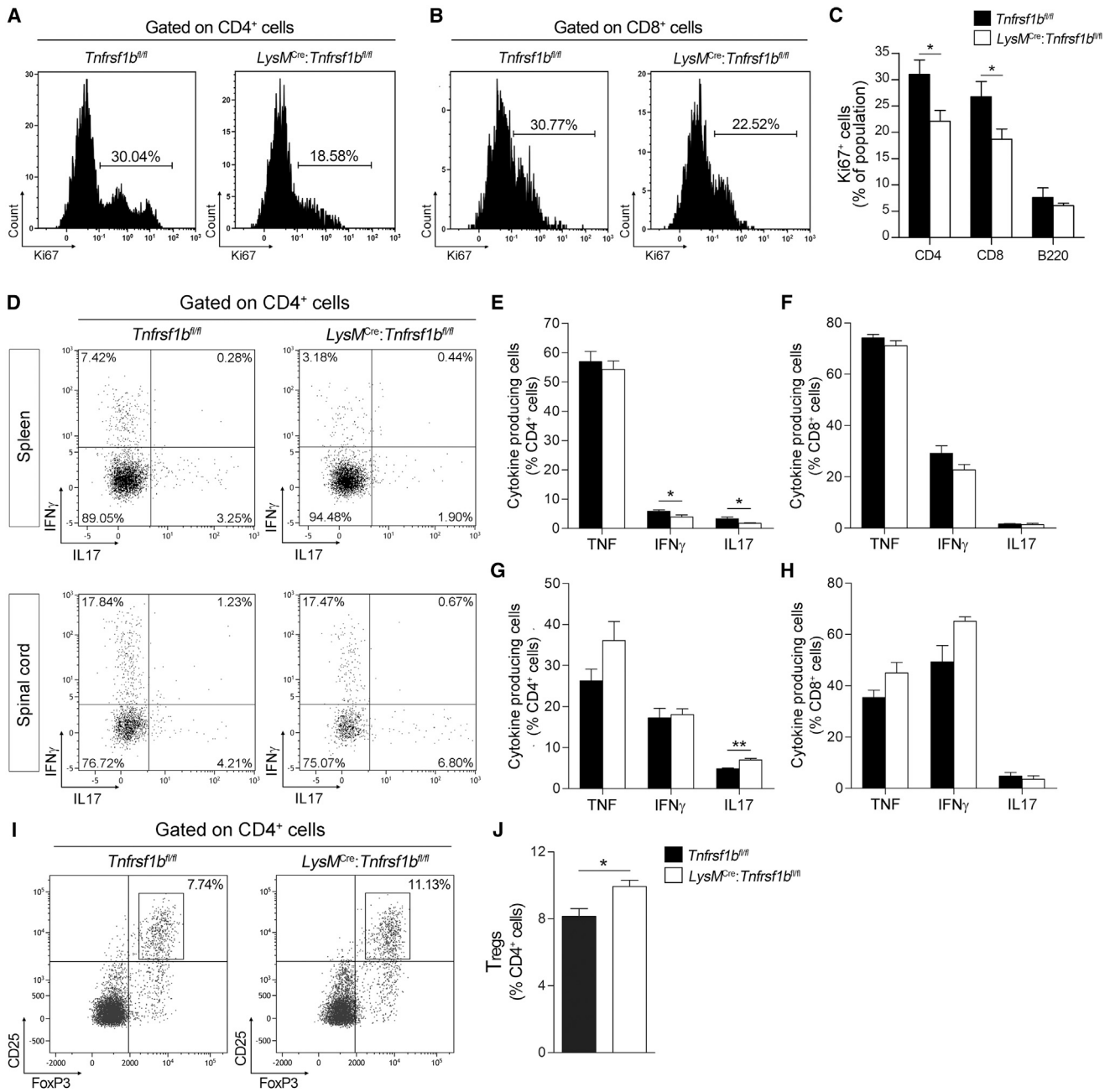


Figure 6. Ablation of TNFR2 in *LysM*^{Cre}:*Tnfrsf1b*^{fl/fl} Mice Results in Impaired T Cell Proliferation and Effector Function in the Spleen after EAE
(A and B) Representative flow plots of Ki67 expression in CD4 (A) and CD8 (B) T cells in *Tnfrsf1b*^{fl/fl} and *LysM*^{Cre}:*Tnfrsf1b*^{fl/fl} mice at 12 dpi.
(C) Quantification of Ki67⁺ splenic lymphocytes. n = 4–5/group; *p ≤ 0.05, Student's t test.
(D) Representative flow plots of cytokine expression in CD4 T cells from spleen and spinal cord at 20 dpi.
(E–H) Quantification of cytokine expression in CD4 (E, G) and CD8 (F, H) T cells; n = 3–6/group; *p ≤ 0.05, **p ≤ 0.01, Student's t test.
(I) Representative flow plots of splenic T_{reg} in *Tnfrsf1b*^{fl/fl} and *LysM*^{Cre}:*Tnfrsf1b*^{fl/fl} mice at 20 dpi.
(J) Quantification of splenic T_{reg} frequency. n = 4–5/group; *p ≤ 0.05, Student's t test.
All graph bars represent SEM. See also Figure S6.

paralleled by a higher CNS influx of activated immune cells. This indicates that TNFR2-dependent signals in microglia suppress neuroinflammation, and without them the CNS is more vulnerable to immune-inflammatory attack from the periphery. As

EAE progresses, the clinical profiles of the two genotypes converge, and this may be due to more immune cells flooding the CNS and overcoming the antiinflammatory “barrier” set by microglial TNFR2. Nevertheless, our data underscore the

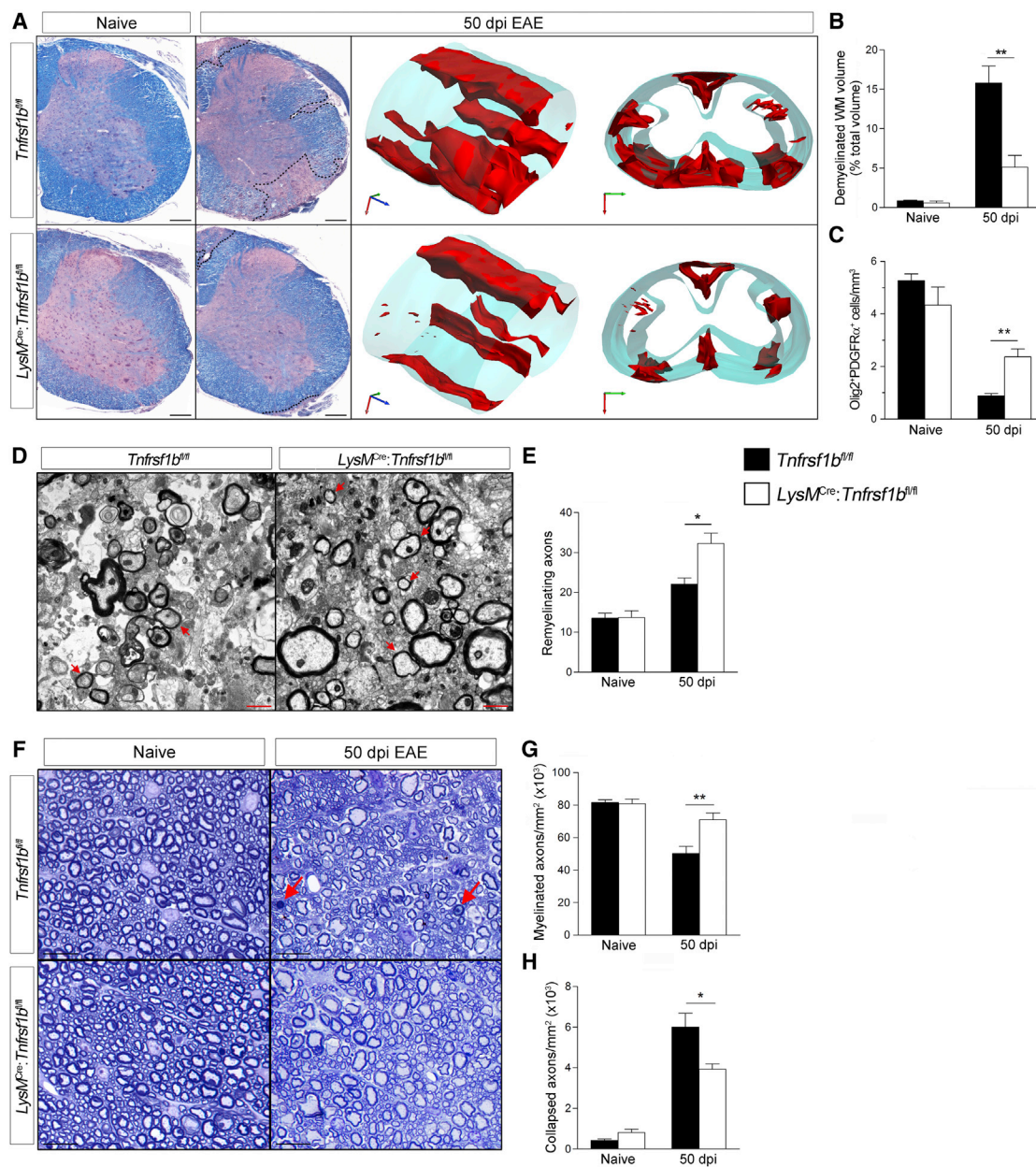


Figure 7. Ablation of TNFR2 in *LysM^{Cre}:Tnfrsf1b^{fl/fl}* Mice Results in Reduced Demyelination and Improved Remyelination and Neuroprotection in EAE

(A) Assessment of demyelination in the spinal cord of *Tnfrsf1b^{fl/fl}* and *LysM^{Cre}:Tnfrsf1b^{fl/fl}* mice by LFB staining. Left: contoured areas show regions of demyelinated white matter. Scale bars, 200 μ m. Right: 3D reconstructions of the demyelinated areas (red).
 (B) Quantification of the demyelinated white matter volume. $n = 3\text{--}5/\text{group}$; $**p \leq 0.01$, Student's t test.
 (C) Quantification of Olig2⁺PDGFR α ⁺ OPCs in the spinal cord. $n = 3\text{--}5/\text{group}$; $**p \leq 0.01$, Student's t test.
 (D) Representative electron micrographs of the spinal cord at 50 dpi showing remyelinated axons (red arrows). Scale bar, 1 μ m.
 (E) Quantification of remyelinated axons. $n = 4\text{--}5/\text{group}$; $*p \leq 0.05$, Student's t test.
 (F) Toluidine blue staining of spinal cord sections from naive and 50 dpi mice. Red arrows indicate degenerated axons. Scale bar, 10 μ m.
 (G and H) Quantification of myelinated (G) and degenerated (H) axons. $n = 4\text{--}5/\text{group}$; $*p \leq 0.05$, $**p \leq 0.01$, Student's t test.
 All graph bars represent SEM.

protective role of microglial TNFR2 in the early stage of disease, which seems to participate in the immediate response of the CNS to alterations of its homeostasis.

To gain insight into the mechanisms of microglial TNFR2-dependent regulation of EAE, we performed RNA-seq analysis of the microglial transcriptome of *Cx3cr1^{CreER}:Tnfrsf1b^{fl/fl}* and

Tnfrsf1b^{fl/fl} mice at disease onset. The picture that emerged is twofold. First, TNFR2 is crucial for microglial homeostatic functions. For example, TNFR2 ablation causes downregulation of the microglia-specific gene *Trem2*, which is necessary for innate immunity, phagocytosis, and resolution of inflammation (Neumann and Takahashi, 2007). Loss-of-function mutations of *Trem2* in humans lead to neurodegenerative disease, including a form of late-onset Alzheimer's disease (Guerreiro et al., 2013; Jonsson et al., 2013), where the *Trem2* mutation has been suggested to disrupt microglial interaction with damaged neurons preventing their phagocytosis (Walter, 2016). Our in vitro data suggest that *Trem2*-dependent phagocytosis may be regulated by TNFR2. Indeed, without TNFR2, phagocytosis is drastically impaired, as is the expression of *Trem2*. The impaired ability of TNFR2-deficient microglia to respond to danger signals is reflected in the altered expression of the sense genes, especially those required for detection of endogenous signals. This includes P2 purinergic receptors, which respond to ATP released by degenerating cells (Rodrigues et al., 2015), and Siglecs that keep microglia in a silent homeostatic status (Linnartz-Gerlach et al., 2014). Together, these data indicate that TNFR2 is an important signal for host defense and proper microglial response to injury.

The second key finding of the RNA-seq analysis is that TNFR2-ablated microglia display a more invasive and proinflammatory phenotype. For instance, genes encoding cell adhesion molecules were upregulated in microglia lacking TNFR2. One example is *Vcam-1*. VCAM-1⁺ microglial cells have been identified at the edges of MS lesions in the proximity of oligodendrocyte cell bodies where oligodendrocyte loss occurs, suggesting that this contact may be detrimental to their survival (Peterson et al., 2002). Integrins (*Itgal*, *Itga4*) were also upregulated, and because they are known to modulate microglial migration and activation during neuroinflammation (Milner and Campbell, 2002), this further supports the idea that lack of TNFR2 exacerbates the microglial proinflammatory phenotype. This is also indicated by the upregulation of proinflammatory genes, such as chemokines. The elevated chemokine production by TNFR2-deficient microglia can help explain the increased influx of immune cells in *Cx3cr1*^{CreER}:*Tnfrsf1b*^{fl/fl} mice. This may also be driven by a concomitant increase in BBB permeability, which is suggested by the early downregulation of ZO-1 expression. ZO-1 is decreased as early as 12 days after EAE induction, indicating that BBB alterations dependent on dysregulated TNFR2-ablated microglia could indeed be one of the earliest, and perhaps most critical, drivers of the exacerbated pathology in *Cx3cr1*^{CreER}:*Tnfrsf1b*^{fl/fl} mice. In addition, the upregulation of vascular endothelial growth factor A (*Vegfa*) found in the RNA-seq screening also supports the idea of a more permeable BBB. Indeed, several reports have shown that CNS-derived VEGFA is implicated in BBB disruption, because its ablation prevents BBB breakdown and lymphocyte influx in EAE (Argaw et al., 2009, 2012). Another interesting finding from the RNA-seq analysis is the upregulation of *Hgf* in microglia of *Cx3cr1*^{CreER}:*Tnfrsf1b*^{fl/fl} mice. HGF has been described as the primary microglial-derived chemotactic factor for OPCs, promoting their proliferation and migration to the site of demyelination (Lalive et al., 2005). This fits well with our data showing

increased presence of OPCs in *Cx3cr1*^{CreER}:*Tnfrsf1b*^{fl/fl} mice with chronic disease, where *Hgf* may serve as a reparative mechanism.

For a full understanding of myeloid TNFR2 function, the next step was to address the role of TNFR2 in peripheral Mo/MΦs, for which we used *LysM*^{Cre}:*Tnfrsf1b*^{fl/fl} mice. Because infiltrating monocytes and resident microglia both differentiate into macrophages that contribute to demyelination in EAE, the expectation was for monocyte/macrophagic TNFR2 to engage processes similar to those in microglia, resulting in worsening of the clinical outcome as in *Cx3cr1*^{CreER}:*Tnfrsf1b*^{fl/fl} mice. Unexpectedly, our data showed the opposite, with *LysM*^{Cre}:*Tnfrsf1b*^{fl/fl} mice being protected from EAE because of their inability to mount an efficient autoimmune response. This was dependent on impairment of two key steps in T cell activation: expansion and differentiation. Mo/MΦs can regulate these processes by presenting antigens and secreting cytokines (Hume, 2008; Wang et al., 2015) such as transforming growth factor β1 (TGF-β1) and IL-6. The concerted action of TGF-β1 and IL-6 drives T cell expansion and also differentiation from naive to a Th17 phenotype (Veldhoen et al., 2006), which is the key encephalitogenic population in EAE. Because both phenomena are suppressed in *LysM*^{Cre}:*Tnfrsf1b*^{fl/fl} mice, it is possible that TNFR2 regulates the expression of these cytokines in Mo/MΦs. This idea is supported by our RNA-seq data, where we saw a reduction of both TGFβ1 and IL-6 signaling in TNFR2-deficient microglia. If this occurred also in Mo/MΦs, it would suggest that the opposite functions of microglial versus monocyte/macrophagic TNFR2 may depend not necessarily on the activation of distinct pathways, but on the engagement of the same pathways that lead to opposite effects because of the different compartments from where the cells originate.

TNFR2 in Mo/MΦs could also be regulating T cell expansion and differentiation indirectly by affecting regulatory T cells. T_{reg} frequency is increased in *LysM*^{Cre}:*Tnfrsf1b*^{fl/fl} mice, and their powerful suppressive activity may contribute to dampening T cell effector function in EAE. In addition to these mechanisms, we cannot exclude the possibility that macrophagic TNFR2 may act via B cell modulation. Indeed, we showed that *LysM*^{Cre}:*Tnfrsf1b*^{fl/fl} mice have reduced MHCII-expressing B cells in the spleen. Their compromised antigen presentation capacity could be in part responsible for the reduced EAE severity.

As far as tmTNF-expressing cells that interact with microglia and Mo/MΦs to carry out TNFR2 functions, they may differ in physiological and pathological conditions. In the normal CNS, microglial TNFR2 will exert its beneficial homeostatic functions likely via contact with tmTNF-expressing microglia and astrocytes. In injury conditions, the repertoire of possible microglial TNFR2 activators widens as all CNS cells (including neurons) and infiltrating immune cells upregulate TNF production (Probert, 2015). Microglia, however, remain the most efficient TNF producers (Olmos and Lladó, 2014), suggesting that much of the tmTNF-TNFR2 protective signaling may be driven by a microglial cell-autonomous process both under physiological and pathological conditions. In the periphery, TNFR2-expressing Mo/MΦs may encounter their tmTNF ligand on virtually all leukocytes, particularly in disease conditions, but also on endothelial and stromal cells, such as fibroblasts and pericytes. Among

leukocytes, dendritic cells may be important partners of TNFR2-expressing Mo/MΦs, because they have been shown to play important roles in innate immune regulation precisely via tmTNF (Xu et al., 2007).

Overall, our study fits within the now accepted model that in pathological conditions the contribution of microglia and peripheral myeloid cells to disease etiology, progression, and resolution may diverge (Shemer and Jung, 2015). In EAE, for example, Yamasaki and colleagues elegantly showed that peripherally derived macrophages associate with nodes of Ranvier and initiate demyelination, whereas microglia take on a protective function by clearing cellular debris (Yamasaki et al., 2014). In this context, we propose TNFR2 as one of the possible effectors of this dual behavior, because TNF production and TNFR2 expression are highly upregulated following EAE in myeloid cells.

Finally, our work further highlights the complexity of TNF function in neuroimmune disease. Not only does TNF have opposite roles whether in soluble or transmembrane form, with sTNF being proinflammatory and tmTNF protective, but so does its receptor TNFR2 depending on the location in central or peripheral myeloid cells. This must be taken into account from a clinical perspective, because strategies enhancing TNFR2 signaling have been proposed as therapeutic avenues in neurodegenerative diseases (Dong et al., 2016; Maier et al., 2013). TNFR2 agonists specifically targeted to the CNS via ad hoc delivery systems at the appropriate time may be a viable pharmacological approach provided their effects on peripheral immune activation are minimized.

EXPERIMENTAL PROCEDURES

Mice

Adult (2–4 months) female and male mice were used in this study. Mice with conditional ablation of the TNFR2 gene (*Tnfrsf1b*) were generated by crossing *Cx3cr1*^{CreER;EYFP+/-} (Jackson Laboratory, 021160) and *LysM*^{Cre+/-} mice (Jackson Laboratory, 004781) with *Tnfrsf1b*^{fl/fl} mice. *Tnfrsf1b*^{fl/fl} mice were obtained by breeding C57BL/6NTac-*Tnfrsf1b*^{tm1a(EUCOMM)Wtsi/lcs} mice (European Mouse Mutant Archive) with an Flp recombinase (FLP) deleter (Jackson Laboratory, 009086) to remove the flippase recognition target (FRT)-flanked neomycin cassette. In all experiments, *Tnfrsf1b*^{fl/fl} littermates were used as controls. In *Cx3cr1*^{CreER;Tnfrsf1b}^{fl/fl} mice, Cre recombinase was induced by five daily intraperitoneal (i.p.) tam injections (2 mg/mouse/day) followed by a 28-day waiting period. Control mice received the same treatment. *Rosa26*^{tdTomato+/-} reporter mice (007914) and CD45.1 congenic C57BL/6 mice (002014) were obtained from Jackson Laboratory. Colonies were housed in the virus/antigen-free Animal Core Facility of The Miami Project to Cure Paralysis, with a 12-hr light-dark cycle, controlled temperature and humidity, and were provided with water and food ad libitum. Experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of the University of Miami.

Behavioral Assessments

Open-field and Rotorod tests were performed as previously described (Madisen et al., 2016a).

Induction of Experimental Autoimmune Encephalomyelitis

Active EAE was induced with myelin oligodendrocyte glycoprotein (MOG_{35–55}) peptide as previously described (Brambilla et al., 2014).

Isolation of Leukocytes from Spinal Cord and Spleen

Cells were isolated as previously described (Brambilla et al., 2014).

Bone Marrow Transplantation

Bone marrow transplantation was performed as previously described (Ashbaugh et al., 2013).

Luxol Fast Blue Staining and Quantification of Demyelinated White Matter Volume

Paraformaldehyde (PFA)-fixed segments of the spinal cord were paraffin embedded, sectioned into 15-μm-thick cross sections with a Leica RM 2135 microtome, and stained with Luxol fast blue (LFB) and H&E. Twenty serial sections at 120 μm intervals were used to estimate the demyelinated white matter volume. Demyelinated areas were outlined with an Olympus BX51 microscope, and demyelinated white matter volume was quantified with StereoInvestigator software (MicroBrightfield). 3D reconstructions of the demyelinated spinal cord were performed on the same serial sections with NeuroLucida software (MBF Bioscience).

Toluidine Blue Staining and Electron Microscopy Tissue Preparation

Toluidine blue staining and electron microscopy tissue preparation were performed as previously described (Brambilla et al., 2014).

Statistical Analysis

Statistical analysis of EAE clinical course was carried out with the Mann-Whitney *U* test. For analysis of RNA-seq data, see Supplemental Experimental Procedures. All other data were analyzed by one-way ANOVA followed by Tukey test for multiple comparisons. In single comparisons, Student's *t* test was applied. The *p* values ≤ 0.05 were considered statistically significant. Data were expressed as the average of multiple determinations ± SEM. Statistical analyses were performed with Prism software.

For all other methods, protocols, and materials, see Supplemental Experimental Procedures.

ACCESSION NUMBERS

The accession number for the sequencing data reported in this paper is GEO: GSE78082.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and three tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2016.11.083>.

AUTHOR CONTRIBUTIONS

H.G. conducted experiments, analyzed data, and drafted the manuscript. M.C.D., J.L.B., and V.P.L. analyzed the RNA-seq data. C.S.C. did stereological counting. M.T. did western blotting and colony management. C.D.-H. did microglia cultures and colony management. D.G.E. and K.L.L. analyzed data; P.M.M. conducted behavioral tests. R.B. conceived the study, conducted experiments, analyzed data, and wrote the manuscript. All authors reviewed and edited the manuscript.

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